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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of
Bright et al.

Appln. No. 08/374,783 Group Art Unit: 1809

Filed: September 6, 1995 Examiner: A. Wang

For : IMPROVED PLANT GERMPLASM

DECLARATION OF DIDIER THOMAS

I, **Didier Thomas** hereby declare that:

1. I am a Belgian citizen residing at 56 Wilwood Road, Bracknell,Berkshire, UK.

2. In 1991, I graduated from the Catholic University of Louvain (Belgium) with a masters degree in "Engineering and Agronomical Sciences" with the highest honour (PGD). I also received a PhD degree in "Agronomical Sciences and Biological Engineering" with the highest honour (PGD), from the Catholic University of Louvain in 1998. Since 1994, I have been employed at the Jealott's Hill International Research Centre of Zeneca Agrochemicals which is located in Bracknell, Berkshire in the United Kingdom. I am currently a research scientist in the Plant Biotechnology Department, supervising and conducting research in gene regulation in plants.

3. I am familiar with the content of the above-identified patent application. I have reviewed the prosecution history and, I understand that there is an objection that the specification does not enable a skilled person to work the invention across the full range claimed, in particular, because the range of developmental promoters and disrupter genes illustrated in the application and also in the declaration of Ian Jepson, which was filed in support of the application and which I have also read is limited.

4. I can confirm that under my direct supervision or control, the experiments described below were carried out. These experiments demonstrate that germination inhibition can be achieved using developmental promoters other than the malate synthase (MS) promoter illustrated previously. In the study described, we used a different control region obtained from a plant cysteine protease (CP) gene to replace the MS promoter in the original Popeye constructs (see Figure 8 of application). The progeny seed of transgenic plants containing an expression cassette comprising the ribonuclease gene barnase under the control of the CP promoters clearly showed impaired germination. This further illustrates that a range of tightly regulated developmental promoters can be used successfully to drive cytotoxic genes in germplasm protection strategies.

The work also demonstrates that germination inhibition can be switched off following safener application during seed development. This results in an accumulation of barstar within the seed, which protects it from the damage barnase would otherwise produce during germination.

5. Cysteine proteases (CPs) are encoded by a multi-gene family and play a key role in seed storage mobilisation during germination. A reverse transcriptase polymerase chain reaction (RT-PCR) strategy was applied to RNA from three-day-old oilseed rape seedlings, using primers designed from heterologous CP sequences. Three classes of CP clones, belonging to the papain superfamily, were isolated. RNA blot analysis indicated that the three classes are expressed in cotyledons, a few days after seed imbibition. Apart from class 1, which is also expressed in flower buds, transcripts were not detected in the seed or mature plant organs. A cDNA library was constructed from two-day-old oilseed rape seedlings and seven full length cDNAs were identified and sequenced.

6. The presumed promoter region of one representative per class was isolated from a genomic library and characterised by sequence analysis and primer extension. To gain more detailed information on the transcriptional regulation of the three classes

of genes, regulatory regions were fused to a reporter gene (β-glucuronidase) and analysed in transgenic tobacco. High expression levels were found from the class 1 (CP12) promoter during pollen germination and very low levels during seed germination. In contrast, class 2 (CP25) and 6 (CP66) promoters were active in a range of tissues. CP25 directed high levels of reporter-gene expression mainly in the radicle, endosperm and cotyledons of five-day-old tobacco seedlings. The CP66 promoter was moderately active in the endosperm of germinating seeds and in the crown area, at the interface between radicle and hypocotyl. No detectable expression was found from CP66 in the radicle, cotyledons or mature plant tissues. However, both CP25 and CP66 promoters were found to be deregulated in calli and to a lesser extend in pollen. I would expect that ectopic expression from the promoters could be minimised by deletion.

7. Two new germplasm protection constructs were built using the CP25 and CP66 germination-specific promoters fused to the barnase gene to create PopeyeIV-25 and PopeyeIV-66, respectively. In PopeyeIV-66, the TMV-omega translational enhancer was introduced after the CP66 promoter in order to boost barnase expression. Both constructs also comprised a barstar gene under the control of the maize GST promoter for germination recovery and a barstar gene driven by a bacterial promoter to protect *E. coli* and *Agrobacterium* from any leakage of barnase expression during the cloning/transformation steps. The overall cloning strategy is illustrated in Figure 1 (Annex A). Tobacco plants were transformed with PopeyeIV-25 or PopeyeIV-66 constructs using *Agrobacterium*-mediated transformation. The presence of the transgene was confirmed by PCR analysis and, for each construct, 40 PCR-positive lines were grown to maturity in the glasshouse and self-pollinated.

8. The phenotype of the primary transformants was normal which confirms that both promoters are tightly controlled during vegetative growth. The ability of segregating progeny seed (T1 population) to undergo normal germination and develop into healthy seedlings was assessed by sowing two batches of fifty seeds on ½ Murashige-Skoog (½ MS) media. The pictures of three lines per class which showed

impaired germination by comparison with the control are provided (Figure 2, Annex A).

In PopeyeIV-25 the presence of the transgene resulted in a slight reduction in germination rate and in a general slow down of early seedling growth, possibly by interfering with the mobilisation of food storage from the cotyledon to the leaf. During germination, CP25 activity mainly occurs in the cotyledons and in a lesser extend in the radicle. The radicle expression is limited to the vascular system, therefore barnase expression in this tissue is likely to be compensated by the production of barstar under the control of the GSTII-27 inducible promoter. The GST promoter is indeed deregulated in the radicle which leads to constitutive expression at that stage and results in prevention of barnase damage.

In PopeyeIV-66 the presence of the transgene resulted in a dramatic reduction in germination rate, in a general slow down of germination and in radicle abortion in the most extreme cases. This phenotype is consistent with the pattern of expression from the CP66 promoter when fused to the GUS reporter gene (Figure 3, Annex A). CP66 activity during germination only occurs in the dying endosperm and at the level of the crown, at the interface between the radicle and the hypocotyl. Although some seedlings manage to grow through by producing an alternative root system, the gap in development compared to the wild type is not closed during the early seedling growth (Figure 4, Annex A).

9. The Popeye IV-66 system was further characterised in an attempt to recover normal germination following activation of the GST inducible promoter. Cuttings were obtained from line 6.16 as well as wild type tobacco plants and grown to maturity in the glass-house (3 inch pots, compost). Half of the plants were treated by root drenching every three days from the onset of flowering to the end of seed maturation using 40 ml of a 0.5 g/L solution of the safener, 3-dichloroacetyl-2,2,5-trimethyl-1,3-oxaxolidone, in an attempt to accumulate barstar in the seed prior to germination. The other half of the plants were root drenched with water as a control experiment. Each inflorescence was bagged to ensure self pollination and watered daily between treatments.

Seed from treated and untreated plants was sown on ½ MS media (50 seed/plate) to compare germination rates and seedling fitness. Seed from untreated 6.16 plant showed a 1:4 segregation (24%) based on their ability to germinate, as expected from a single loci line expressing barnase. Seed from treated 6.16 plant had comparable germination rates to the wild type (99%) thereby demonstrating that germination inhibition can be switched off following chemical treatment (Figure 5, Annex A).

The phenotype of seedlings (Figure 6, Annex A) and young plants (Figure 7, Annex A) grown from seed that had accumulated barstar during seed maturation (induced) was similar to that of wild type plants.

10. The experiments described above clearly demonstrate that impaired germination can be achieved by expressing a cytotoxic gene under the control of a germination-specific promoter other than MS. To our knowledge it is also the first time that in such system, reversal of the cytotoxic gene effect and normal germination was achieved following plant induction using a chemical registered for use in agriculture. Moreover, in our experiments, plant induction with the safener was performed during seed development. This offers several advantages over systems where seed would be coated with the switch chemical or seedlings treated in the farmer's field. Plant induction in the production field (as opposed to farmer's field) benefits from smaller acreage, easily controllable treatment conditions and the possibility to quality check seed batches for germination efficiency before commercialisation.

11. As regards the use of disrupter genes other than barnase, it is clear from a review of the literature that these are available and there is, in my view, no reason why any of the known disrupter genes would not be operable in the systems described in the present application. WO97/04116 (Annex B) reports work on such systems which was carried out at Zeneca Agrochemicals. This publication describes how four different disrupter genes may all be used in the sort of systems claimed in the present application. They consist of the T-urf13 gene, genes encoding an α- or β- tubulin, a short sense construct to the adenine nucleotide translocator (ANT) and short sense co-

surppression of two essential maize cell cycle genes, cdc25 and replication origin activator (ROA) of the inner mitochondrial membrane. The examples, and in particular Examples 2, 6 and 8 indicate that these disrupter genes, when transformed into maize cells, inhibit cell proliferation.

Other examples can be found in one of our pending application PCT/GB99/02699 (a copy of which is attached as Annex C) where a protease is used as an alternative to barnase ribonuclease. In house experiments demonstrated that expression of a plant cysteine protease into maize cells, inhibits cell proliferation. Proteases can be inactivated by covalent or non-covalent binding from a range of inhibitory proteins, in a way similar to the barnase/barstar system. Cysteine protease propeptides, in particular, have been shown to be potent selective inhibitors of their cognate protease in the nano-molar range (Carmona *et al.*, Annex D).

12. As regards the use of inducible systems other than the GST switch, a range of systems are currently under development for plants (Jepson *et al.*, (1998) Chemical-inducible gene expression systems for plants: a review. *Pestic. Sci.* **54**, 360-367 - Annex E). In particular, it is clear from a review of publications describing a range of successful applications of the Alc switch, that this system can only be more effective than the GST switch for recovering normal germination (Caddick *et al.* (1998) An ethanol inducible gene switch for plants used to manipulate carbon metabolism. *Nature Biotech.* **16**, 177-180 (Annex F), Jepson *et al. supra.* (Annex E), Salter *et al.*, (1998) Characterisation of the ethanol inducible alc gene expression system for transgenic plants. *The Plant J.* **16**, 127-132 (Annex G)). Therefore, there is, in my view, no reason why after optimisation, any of the known switch systems would not be operable in the systems described in the present application.

13. Taken all together, the above data and the attachments are, in my view, indicative of the fact that the germplasm protection system of US Serial No. 08/374,783 has a wide range of workable applications and should not be restricted to a specific combination of barnase/barstar genes under the control of the MS and GST promoters respectively.

Here ends my declaration.

Didier Thomas

10th January 2000
Date